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ScienceDirect
Virus Research 129 (2007) 26–34
Virus Research
www.elsevier.com/locate/virusres

Identification of a novel virus in pigs—Bungowannah virus: A possible new species of pestivirus

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Received 2 February 2007; received in revised form 16 March 2007; accepted 2 May 2007
Available online 11 June 2007

Abstract

In 2003 an outbreak of sudden deaths occurred in 3–4-week-old piglets on a farm in New South Wales, Australia. There was a marked increase in the birth of stillborn foetuses. Pathological changes consisted of a multifocal non-suppurative myocarditis. A viral infection was suspected but a wide range of known agents were excluded. A modified sequence independent single primer amplification (SISPA) method was used to identify a novel virus associated with this outbreak. Conserved 5′UTR motifs, the presence of a putative N^{pro} coding region and limited antigenic cross-reactivity with other members of the *Pestivirus* genus, support the placement of this virus in the *Pestivirus* genus. Phylogenetic analysis of the 5′UTR, N^{pro} and E2 coding regions showed this virus to be the most divergent pestivirus identified to date.

Keywords: Bungowannah virus; Pestivirus; Porcine myocarditis syndrome

1. Introduction

The *Pestivirus* genus of the family *Flaviviridae* includes viruses that infect wild and domestic ruminants and pigs. While four species are currently recognised by the International Committee on Taxonomy of Viruses (Thiel et al., 2005), there are claims for the recognition of three additional species isolated from giraffe (Avalos Ramirez et al., 2001), 'HoBi' virus from foetal calf serum (Schirrmeier et al., 2004) and a virus from pronghorn antelope (Vilcek et al., 2005). This report describes a novel pestivirus that was identified in pigs and warrants consideration as a new species in this genus.

In June 2003 an outbreak of disease was reported on a pig farm in New South Wales, Australia. The outbreak initially presented as sudden death in 3–4-week-old weaning age piglets, but soon after the onset there was a marked increase in the birth of stillborn foetuses and a slight increase in the occurrence of mummified piglets. Pathological changes in affected animals consisted almost exclusively of a multifocal non-suppurative myocarditis with myonecrosis observed in some cases. There was evidence of secondary congestive cardiac failure in some piglets. The clinical presentation was referred to as the porcine myocarditis (PMC) syndrome. Over a period of several months, the disease gradually spread through the large pig-farming complex until pigs in all production modules had been affected. Clinical evidence of disease resolved on most production units within 3–6 months. There was evidence for the involvement of an infectious agent, most probably viral, but extensive investigations excluded a wide range of known agents (McOrist et al., 2004). While virus isolation attempts were unsuccessful, inoculation of clinical material into porcine foetuses provided evidence of transmission of an infectious agent (D. Finlaison, unpublished data). Sequence independent single primer amplification (SISPA) has been utilised to identify novel viral nucleic acid in serum samples (Allander et al., 2001; Jones et al., 2005). A modification of this method was used to identify a novel virus associated with this outbreak of porcine myocarditis.

This paper describes the identification and preliminary characterisation of this virus. Conserved 5′UTR motifs, the presence of a putative N^{pro} coding region and limited antigenic cross-reactivity with other members of the *Pestivirus* genus, support the placement of this virus in the *Pestivirus* genus. However, phylogenetic analysis of the 5′UTR, N^{pro} and E2 coding regions shows this virus to be the most divergent pestivirus identified

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doi:10.1016/j.virusres.2007.05.002

to date. This evidence suggests that this novel virus represents a new species within the *Pestivirus* genus. Finally, the name "Bungowannah virus" is proposed for this virus.

2. Materials and methods

2.1. Specimens

SISPA was performed on a pool of 12 sera collected from a litter of experimentally infected pig foetuses euthanased 13 days after inoculation. Four of these foetuses had been inoculated at 92 days of gestation with a 20% (v/v) homogenate of myocardium and lung from foetuses considered likely to be naturally infected by the agent causing the PMC syndrome. The four inoculated foetuses had mild to moderate increases in serum IgM levels and equivocal increases in IgG levels. One of the uninoculated foetus had an equivocal increase in IgM but none of the uninoculated foetuses had developed an increase in IgG at the time of euthanasia (data not presented). However, crystalline arrays of virus-like particles were observed in the heart tissue of nine foetuses from this litter, suggesting in utero transmission of an infectious agent. Because not all foetuses had mounted a humoral immune response it was considered likely that some foetuses may be viraemic. A serum pool was selected for SISPA in preference to a pool of the heart tissues due to the low levels of host DNA and RNA in serum compared to tissue extracts.

2.2. Sequence independent single primer amplification (SISPA)

The amplification of nucleic acid from virions in serum was based on the method of Allander et al. (2001) with several modifications aimed at reducing the quantity of host derived cellular RNA, maximising the yield and length of the amplified nucleic acid and minimising template-independent side reactions (Baugh et al., 2001). Pooled serum (120 μL) was diluted 1 in 3 with phosphate buffered saline (PBS) pH 7.3 and filtered through a 0.22 μM filter (Ultrafree MC, Millipore) by centrifugation at 2000 × *g*. DNase I (250 U; Stratagene) and an RNase Cocktail (0.5 U RNase A and 20 U RNase T1; Ambion) were incubated with the 360 μL of diluted serum at 37 °C for 2 h. The treated sample was then divided into two aliquots for DNA and RNA extraction.

RNA was extracted from a 180 μL aliquot of diluted serum and eluted in 20 μL of RNase free water using an RNeasy minikit (Qiagen). RNA (9 μL) was mixed with 10 pmol of random hexamers (Stratagene) to make a final volume of 10 μL. Double stranded RNA was separated by heating the mixture at 90 °C for 3 min and cooling to 4 °C. First strand synthesis was carried out at 50 °C for 1 h in a final volume of 20 μL, containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.2 M dTT, 10 mM dNTP mix, 400 U Superscript III (Invitrogen) and 5 μg T4gene32 protein (USB). Upon completion, the first strand reaction was heat inactivated for 10 min at 70 °C. The second strand cDNA synthesis reagents [20 mM Tris–HCl (pH 6.9), 4.6 mM $MgCl_2$, 90 mM KCl, 0.15 mM β-NAD+, 10 mM $(NH_4)_2SO_4$, 30 mM dNTP mix, 40 U DNA polymerase (Roche Diagnostics), 10 U *E. coli* DNA ligase (Invitrogen), 2 U Rnase H (Roche Diagnostics)], were then added to the first strand reaction mixture in a final volume of 150 μL. After 2 h incubation at 16 °C 10 U of T4 DNA polymerase (Roche Diagnostics) was also added and incubated for 15 min at 16 °C. The second strand reaction was then stopped by heating at 72 °C for 10 min.

DNA was extracted from 180 μL of diluted serum using a DNeasy Tissue kit (Qiagen) in accord with the manufacturers instructions. Extracted DNA (50 μL) was mixed with 10 pmol of random hexamers (Stratagene), 5 mM dNTP mix, 5 μg T4gene32 protein (USB), 10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 and 5 U 3′–5′ exo Klenow fragment DNA polymerase (New England Biolabs). Second strand DNA synthesis was carried out at 37 °C for 1 h.

Double stranded DNA, generated using either extracted RNA or DNA as the first strand template, was extracted in phenol:chloroform:isoamyl alcohol (25:24:1, Trace) with spin phase lock tubes (Eppendorf), ethanol precipitated and redissolved in water. Restriction enzyme Csp6.I (10 U, Progen) was then incubated with the DNA for 2 h at 37 °C before heat inactivation at 65 °C for 20 min. CIP dephosphorylase (Roche Diagnostics) and dephosphorylation buffer (10×) were added directly to 50 μL of the digested DNA to give a final concentration of 0.1 U/μL CIP, 0.05 M Tris–HCl and 0.1 mM EDTA, pH 8.5, in a final volume of 60 μL. The mixture was then incubated at 37 °C for 30 min. Another 6 U of CIP dephosphorylase (0.3 μL) was added and incubation continued for another 30 min. After another purification using phenol:chloroform:isoamyl alcohol (25:24:1, Trace) with spin phase lock tubes (Eppendorf), the DNA was ligated overnight to the phosphorylated adapter NCsp [hybridized oligonucleotides NBam24 (8), P-AGG CAA CTGTGC TAT CCG AGG GAG; and NCsp 11, P-TAC TCC CTC GG] and used as a template for PCR (Roche Diagnostic) with oligonucleotide NBam24, as described by Allander et al. (2001).

2.3. Analysis of SISPA products

The PCR product was run on a 1% agarose gel for 2 h. Both distinct bands and regions containing smearing were cut from the gel as individual segments for gel purification (Qiagen) and ligation into TA cloning vector PCR2.1 (Invitrogen). At least 12 colonies from the Top 10 (Invitrogen) transformation for each band or region cut from the SISPA gel were screened directly in a hot start PCR (Qiagen), using plasmid primers M13r and M13-20 at 12 μM. PCR cycling conditions were denaturation/activation at 95 °C for 15 min, 25 cycles of 95 °C 30 s, 50 °C 30 s, 72 °C 3 min, and a final extension at 72 °C for 5 min. PCR product was run on a 1% agarose gel. PCR products, of different sizes, were selected from each SISPA gel cut out to avoid repeated sequencing of the same cloned PCR products. The selected PCR products were purified (MinElute PCR purification kit, Qiagen) and then sequenced at the Australian Genome Research Facility Ltd., using the M13 reverse priming site in the PCR2.1 vector (Invitrogen).

Table 1
Primers for PCR amplification of the novel virus

SISPA clones	Primer name[a]	Primer sequence (5′–3′)	Nested product size	Coding region[b]
CR39	CR39F[c]	CACATCTAGCAGCAGACTATGA	103bp	E^{rns}
	CR39R	GTACCAGTTGCACCACCC		
	CR39FN	TGAAAAGGATTCACGG		
ER510	ER510F	AAACCGACGAAGTAGACC	114bp	p7
	ER510R	AGACGAGAACATAGTGGC		
	ER510FN	GAAACAGTAAAGCCAACG		
	ER510RN	CTGGTAATCGGAAACATC		
ER62	ER62F	GGGACCGAGGGATACGA	98bp	NS5B
	ER62FN	AGAGGTAATTGGGTAT		
	ER62R	CAGCAGGTTGATTTCTTCAT		
	ER62RN	TTGCCAAGTTTCAC		
ER55	ER55F	AAACCGCCGAAGTAAACC	143bp	p7
	ER55R	CTGGAGCCCTGGTAATGG		
	ER55FN	GACGGGAATGGGTTCA		
	ER55RN	TAGGTGCTTCTTATTGGTAT		

[a] F =forward primer, R = reverse primer, FN = forward nested primer, RN = reverse nested primer.
[b] As subsequently determined when further nucleic acid sequence was available.
[c] Primer CR39F was used for both the first and nested polymerase chain reactions because a suitable forward nested primers could not be located in this region.

2.4. Sequence analysis

Sequence data generated by the SISPA was analysed using Basic Local Alignment Search Tools (BLAST) available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/blast). Nucleotide sequence homology was identified using the "discontiguous MegaBlast" and amino acid homology utilising the translated BLAST search "Blastx".

2.5. Specificity of SISPA products

To confirm the authenticity of the viral nucleic acid sequences generated by the SISPA, a nested RT-PCR (refer to Table 1) was developed for each SISPA-derived sequence. These PCR assays were used to detect target nucleic acid sequences in the serum pools used for SISPA, in the pool of foetal heart and lung tissue used as the primary inoculum in this study and in tissues from field cases of PMC. RNA was extracted from 30 mg of each of the tissue samples using RNeasy kits (Qiagen) according to the manufacturer's instructions, eluted in 30 μL of water and 5 μL used in the PCR. From the purified RNA used for the SISPA, 1 μL was used in the PCR. Reverse transcription and amplification of the resulting DNA was completed using the SuperScript III One-Step RT-PCR System (Invitrogen), with the first round PCR primers (see Table 1) at a concentration of 4 μM. Reverse transcription was performed at 50 °C for 50 min followed by denaturation at 94 °C for 2 min. PCR amplification involved 40 cycles (94 °C 15 s, 50 °C 30 s, 68 °C 30 s) followed by a final extension at 68 °C for 5 min. The nested PCR was performed using a hot start PCR (Qiagen) where 1 μL of the RT-PCR product was used as template and nested primers used at 20 μM. Cycle conditions are as described in (Section 2.3) with the exception of the extension time being 30 s. All products were run on a 1.5% agarose gel.

2.6. Examination of field specimens by PCR

To determine if the viral sequences generated by SISPA were associated with field cases of PMC, a total of nine tissue samples collected from field cases were tested by nested PCR using the CR39 primers (Table 1). Tissues tested included heart, lung and spleen.

2.7. Amplification of viral genome

A number of short nucleic acid sequences of presumptive viral origin were identified by SISPA. Both forward and reverse PCR primers were designed based on each of these short fragments (Table 1). A forward primer from one segment and a reverse primer from an adjacent fragment were incorporated in a RT-PCR assay using nucleic acid extracted from field specimens as template. This process was employed to amplify intervening fragments of nucleic acid and permit determination of additional viral sequence. RT-PCR was carried out as described in Section 2.5 with the exceptions that extension times were longer (2–3.5 min, depending on the size of the expected PCR fragment) and primer concentrations were higher (30 μM). To generate sequence data from the 5′ end of the viral sequence an additional RT-PCR using sense primer 5′-CATGCCCATAGTAGGAC-3′ (Ridpath and Bolin, 1998) and antisense primer 5′-ACCAGTTRCACCAMCCAT-3′ (Vilcek et al., 2005) was performed.

Sequence data from the complete 5′ untranslated region (UTR) was generated using rapid amplification of cDNA ends (RACE, BD), as described by BD Biosciences Clonetech with the following modifications. The virus-specific primer CR24R (5′-TCCCCGAAGCTTGGTTTAAT-3′) was used to generate the cDNA. Hot start PCR (Qiagen) was carried out with primers CR39R (Table 1) and BD Universal Primer A mix (5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAA-

CGCAGAGT-3′ and 5′-CTAATACGACTCACTATAGGGC-3′) with an annealing temperature of 67 °C and extension time of 2 min. The virus-specific primer N^{pro} RS (5′-TTGCTAC-AATCGCCCTTCTT-3′) and BD nested Universal Primer A (5′-AAGCAGTGGTATCAACGCAGAT-3′) were used for the hot start Nested PCR, with an annealing temperature of 55 °C and an extension time 2 min. Nested PCR products were cleaned, cloned and sequenced as described for SISPA products in Sections 2.3 and 2.4.

2.8. Phylogenetic analysis

Comparisons of the 5′UTR were conducted using the nucleotide sequence and the N^{pro} and E2 using the deduced amino acid sequence. Sequences representing the *Pestivirus* genetic groups *BDV*, *BVDV-1*, *BVDV-2*, *CSFV* and the giraffe and pronghorn viruses were obtained from NCBI Genbank as follows:

BDV: Reindeer (gi|20178630); T1802 5′UTR (gi|2138154), N^{pro} (gi|2138154) and E2 (gi|2138154); V2536 5′UTR (gi|2138157), N^{pro} (gi|2138157) and E2 (gi|2138157), X818 (gi|20198946),

BVDV-1: CP7 (gi|1518835); NADL (gi|323205); NY1 5′UTR (gi|548089), N^{pro} (gi|5714458) and E2 (gi|15283984).

BVDV-2: 890 (gi|902376); 21373 (gi|23270352); NY93C (gi|22094502).

CSFV: Alfort (gi|1906629), Brescia (gi|325460), Paderborn 5′UTR (gi|18482493), N^{pro} and E2 (gi|22094503).

"*Giraffe*": H138 5′UTR (gi|7328460), N^{pro} (gi|20178632) and E2 (gi|20178633);

"*Pronghorn*": (gi|59939175).

Comparisons were limited to these viruses because these were the only viruses for which sequence was available in Genbank for each of the three regions of interest. These sequences were compared to the novel viral sequence, using the Phylogeny Inference Package (Version 3.2) in Bionavigator (http://www.angis.org.au/). Multiple alignments for each sequence were completed using ClustalW. The nucleotide alignment for the 5′UTR was run through Puzzle 5.0 to estimate the transition–transversion (Ts–Tv) ratio of the data set and then analysed with DNAdist, using maximum likelihood and the calculated Ts–Tv of 1.42. N^{pro} and E2 amino acid alignments where analysed in the PRODist program (http://www.angis.org.au/). The program Drawtree was then used to generate the dendrograms. Statistical reliability of the dendrograms was determined by bootstrapping the ClustalW alignment 1000 times, using Seqboot, before creating a distance matrix (as described above). Neighbor-joining was used to analyse the distance matrix, which was then run in the Consense program to create the phylogenetic trees. Percentage similarities between the viral sequences were generated using the OldDistance program in the GCG package of Bionavigator (http://www.angis.org.au/).

2.9. Virus isolation

Heart tissue from the 12 foetuses used for SISPA (see Section 2.1) was pooled and a 2% (v/v) homogenate prepared in cell culture medium (Minimum Essential Medium: MEM) without serum but containing antibiotics (1000 units/mL of penicillin and streptomycin, and 4 μg/mL of amphotericin B). The homogenised tissue was clarified by centrifugation at 2000 × *g* for 20 min at approximately 4 °C and the supernatant removed for virus isolation. After removal of the cell culture growth medium, 0.5 mL of the supernatant was adsorbed for 2 h onto 70% confluent monolayers of primary neonatal bovine testis (BT) cells, primary neonatal lamb testis (LT) cells or the continuous porcine kidney cell line PK-15A in 25 cm^2 culture flasks. At the conclusion of the adsorption period, the inoculum was left in the flasks and new complete growth medium (Basal Medium Eagles (BME) supplemented with 10% BVDV free adult bovine serum (BT cells) or MEM supplemented with 10% gamma irradiated foetal bovine serum (LT and PK-15A cells) and antibiotics) was added. After incubation for 5–7 days at 37 °C, the cultures were frozen at approximately −80 °C, thawed and 1 mL of culture fluid passaged to new monolayers. Initially virus replication was monitored by testing of the culture supernatant by nested PCR as described in Section 2.5. Samples of culture supernatant (500 μL) were removed at the conclusion of the adsorption period and after 5 days and $\log_{10}$ dilutions prepared for assay by PCR. Un-inoculated cell cultures were maintained for each cell type and culture supernatant was also removed for assay by PCR. After the development of an appropriate method for fixation of the monolayer and identification of a suitable antiserum, virus replication was monitored by immunoperoxidase staining (see Section 2.10).

2.10. Immunoperoxidase staining

To monitor virus replication and undertake preliminary antigenic characterisation, PK-15A cells were grown in 96-well microplates at a seeding rate of approximately 10^4 cells per well. Cells were inoculated with virus at seeding. Culture fluids were removed from the monolayers after incubation for 4–5 days. The monolayer was washed twice with PBS (pH 7.2), air dried then frozen at or below −20 °C for at least 30 min. The cultures were thawed and fixed in situ for 10 min by adding 100 μL 1.5% (w/v) formaldehyde solution in PBS containing 0.1% (v/v) NP40. The fixative was removed and the cells washed three times with 0.05% Tween 20/water using a microplate washer at the slowest available speed. Immunoperoxidase (IPX) staining was completed as described by Kirkland and MacKintosh (2006), using polyclonal serum (1/200 dilution in PBS containing 1% gelatin) from a pig infected with this novel virus as the primary antibody and peroxidase conjugated goat anti-swine IgG (Jackson Immuno Research) as the second antibody. Serum from a pig on a farm free of PMC was used as a negative control. Once optimal conditions for cell fixation and immunoperoxidase staining had been established, IPX staining was also attempted using monoclonal antibodies (mAbs) that are believed to be pan-reactive against known pestiviruses. Mabs used included 2NB2, P3C12

and P4G11 used as a mixture (Dekker et al., 1995; Shannon et al., 1991) and WB103 and WB105 as a mixture (Edwards et al., 1991). The Trangie strain of BVDV1 was grown in LT or BT cells as a positive control for the IPX staining.

2.11. Serology

Porcine sera were tested for pestivirus group reactive antibodies in an agar gel immunodiffusion test using the *BVDV-1* strain C24V as the antigen and a hyperimmune goat polyclonal antiserum for the reference serum. Serum samples from 45 pigs naturally infected with the novel virus were tested in virus neutralisation (VN) tests for *BDV*, *BVDV-1* and *CSFV*. The VN test method was conducted as described by Kirkland and MacKintosh (2006) and used Australian strains X818 and Trangie for *BDV* and *BVDV-1*, respectively. VN tests for *CSFV* were described by McOrist et al. (2004).

3. Results

3.1. Analysis of SISPA products

A total of 302 RNA and DNA derived clones were sequenced. No sequence with homology to a virus was identified from the DNA SISPA reactions. In contrast, 14 clones derived from the RNA preparations were found to have some sequence homology to viral sequences in Genbank. Eleven clones shared some sequence identity with members of the *Pestivirus* genus and three with porcine endogenous retroviruses. The porcine endogenous retrovirus sequences were considered unlikely to be significant as these sequences are frequently found in normal porcine tissues and cells. Of the 11 clones with some pestivirus sequence homology, four showed homology to the E^{rns} region, three to the p7 region and two each to the NS5A and the NS5B coding regions of the *Pestivirus* genome. Sequence homology with pestiviruses could be identified at the nucleotide level for four of the clones (representing two fragments from each of the E^{rns} and NS5B regions) while seven clones (representing fragments of the NS5A and p7 regions) only showed homology with pestiviruses at the deduced amino acid level. As pestiviruses are known to infect pigs and cause foetal infection and reproductive losses in several species, the significance of these results was investigated further.

3.2. Confirmation of specificity of SISPA products

Three of the four primer groups designed for confirmation of the specificity of the SISPA products (see Table 1) were successfully used to amplify viral nucleic acid from the serum pool used for the SISPA. No detectable product was generated using the primers from the NS5B region.

As the CR39 primers designed against the E^{rns} region had the highest amplification success rate, they were used to test tissues of piglets with clinical signs of PMC for the presence of viral nucleic acid. Seven of the nine samples tested positive as did the pool of four foetal hearts and lungs that was used as the primary inoculum in this study.

Table 2
Comparisons of the identity (%) of the novel virus with representative pestiviruses using the nucleotide sequence for the 5′UTR

	ALFORT	BRESCIA	PADER BORN	X818	T1802	V2536	REIN DEER	NY_93_C	890	1373	CP7	NY_1	NADL	GIRAFFE_H1	PRONG HORN	BUNGO WANNAH
ALFORT	100.0															
BRESCIA	98.7	100.0														
PADERBORN	96.4	96.9	100.0													
X818	78.5	78.4	80.3	100.0												
T1802	79.7	79.7	82.4	96.0	100.0											
V2536	78.4	76.6	79.3	91.4	92.3	100.0										
REINDEER	79.7	79.7	81.5	86.0	88.3	87.4	100.0									
NY_93_C	69.5	69.8	70.0	64.7	66.2	67.1	68.9	100.0								
890	68.6	68.9	69.5	64.7	66.2	67.1	68.9	95.7	100.0							
1373	69.5	69.8	70.0	65.2	66.7	67.6	69.4	99.6	94.8	100.0						
CP7	69.1	70.3	69.1	66.1	65.8	65.8	68.5	75.0	72.8	74.6	100.0					
NY_1	70.0	71.2	69.1	67.0	66.7	66.2	69.4	77.6	75.4	77.2	96.1	100.0				
NADL	71.8	72.1	70.9	69.2	68.9	69.8	71.6	74.7	72.9	74.2	89.5	90.8	100.0			
GIRAFFE_H1	71.8	72.1	71.8	68.3	68.5	70.7	70.7	71.3	69.6	71.3	67.1	68.0	66.8	100.0		
PRONGHORN	60.0	60.9	60.5	62.8	62.8	61.4	60.5	60.0	60.5	60.5	59.5	60.0	63.7	60.5	100.0	
BUNGOWANNAH	59.6	60.8	57.9	53.6	55.9	55.0	56.3	57.0	55.8	56.7	57.5	57.5	60.3	56.1	62.8	100.0

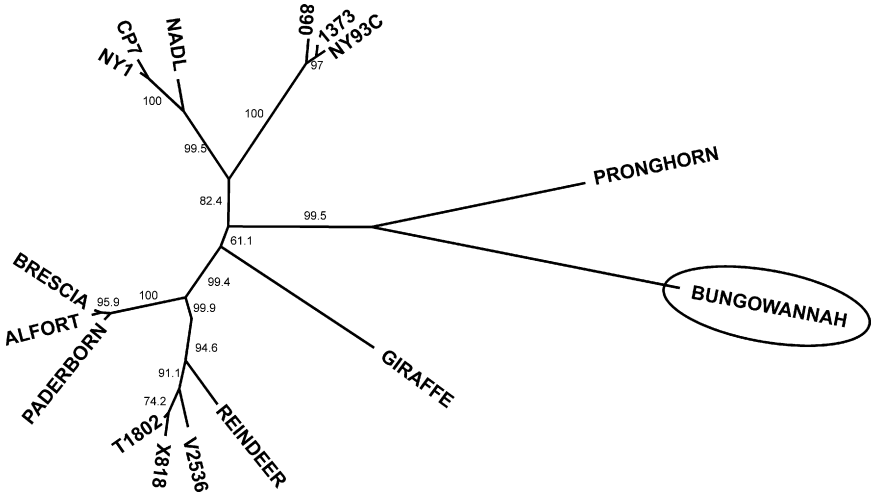

Fig. 1. Dendrogram comparing the genetic diversity of the novel virus with representative pestiviruses using the nucleotide sequence for the 5′UTR. Branch lengths are proportional to genetic distances. Numbers indicate the percentage of 1000 bootstrap replicates that support each labelled interior branch.

3.3. Amplification of viral genome

By using the initial viral sequences and subsequent primer 'walking', additional genome fragments were identified that were consistent with the 5′UTR, N^{pro} and E2 regions of the *Pestivirus* genome. The entire 5′UTR, N^{pro} and E2 regions were subsequently sequenced and used for later phylogenetic analyses. The sequences for these regions have been lodged in Genbank as accession numbers gi|18406774, gi|18406775 and gi|18406777, respectively.

3.4. Sequence analysis

The sequences generated were compared to genomic sequences from the 5′UTR, or the deduced amino acid sequences for the N^{pro} and E2 regions of a number of recognised pestiviruses. The sequence identity of the 5′UTR of the novel virus and viruses from the four recognised pestivirus species (*BVDV-1*, *BVDV-2*, *BDV* and *CSFV*) ranges from 54% to 61% (Table 2). The greatest sequence identity was with the pronghorn virus at 62.8% followed by the giraffe virus at 56.1%. In contrast, the sequence identity among the four recognised species ranges from 64.7% to 82.4% and from 86.0% to 99.6% between viruses from the same species. Fig. 1 illustrates the phylogenetic relationship of the novel virus to representative members of the *Pestivirus* genus.

At the protein level, the identity between the deduced amino acid sequence for the N^{pro} region of novel virus and viruses from the four recognised species of pestivirus ranged from 50.9% to 55.8% (Table 3). The homology is approximately 53–54% between the novel virus and either the pronghorn or the giraffe viruses. The homology between the E2 region of the novel virus and viruses from the four recognised *Pestivirus* species ranged from 47.7% to 51.3% (Table 4). Homology with both the pronghorn and giraffe viruses was approximately 48%. Figs. 2 and 3 illustrate the phylogenetic relationship of the N^{pro} and E2 regions of the novel virus to other isolates of pestivirus at the amino acid level.

3.5. Virus isolation and immunoperoxidase staining

There was evidence of virus replication in each of the BT, LT and PK-15A cell cultures after the primary inoculation although the titres were low, ranging from 4.0 $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$) per millilitre of culture supernatant (PK-15A cells) to approximately 1.5 $\log_{10}$ $TCID_{50}$/mL (BT and LT cells). After further passage in PK-15A cells, titres of approximately 4.0–4.5 $\log_{10}$ $TCID_{50}$/mL were detected but the replication in BT and LT cells diminished to very low or undetectable levels after three passages. The virus has continued to replicate successfully in PK-15A cells for more than 10 successive passages. Immunoperoxidase staining of infected PK-15A cells using the convalescent pig serum revealed intense cytoplasmic staining typical of a non-cytopathogenic pestivirus. No staining was observed with the serum of a pig from an unaffected farm. There was no evidence of staining of cells infected with the novel virus following use of either individual mAbs or pan reactive mAb mixture, despite testing over a wide range of dilutions of the mAbs. In contrast, typical and intense staining patterns were observed with the BVDV infected cultures.

3.6. Serological relationships

Serum from the 45 naturally infected animals showed weak antibody reactions when tested in the BVDV AGID test but had no neutralising activity against a representative strain of *BDV*, *BVDV-1* or *CSFV*.

4. Discussion

In this study we have identified a novel pestivirus associated with myocarditis in pigs. Classification as a pestivirus is based on the presence of a putative N^{pro} coding region and sequence identity in the 5′UTR, N^{pro} and E2 coding regions. The pestivirus with which pigs are most frequently infected is CSFV, although infection with strains of BVDV occurs from time to time (Paton et al., 1992). Four species of pestivirus are currently

Table 3
Comparisons of the identity (%) of the novel virus with representative pestiviruses using the deduced amino acid sequence for the N^{pro} coding region

	ALFORT	BRESCIA	PADERBORN	X818	T1802	V2536	REINDEER	NY_93_C	890	1373	CP7	NY_1	NADL	GIRAFFE_H1	PRONGHORN	BUNGOWANNAH
ALFORT	100.0															
BRESCIA	98.2	100.0														
PADERBORN	96.3	96.9	100.0													
X818	79.6	80.3	81.0	100.0												
T1802	80.9	81.5	81.6	94.5	100.0											
V2536	77.8	77.8	78.5	87.1	88.3	100.0										
REINDEER	77.8	78.4	78.5	82.8	84.1	82.8	100.0									
NY_93_C	77.8	78.4	77.3	75.5	76.1	76.7	75.5	100.0								
890	77.8	78.4	77.2	75.3	75.9	76.5	75.3	99.4	100.0							
1373	77.8	78.4	77.3	74.9	76.1	76.7	74.9	97.6	98.2	100.0						
CP7	75.3	75.9	75.5	76.7	77.3	76.7	76.1	81.6	81.5	81.0	100.0					
NY_1	75.9	76.5	76.1	77.3	77.9	76.1	75.5	79.1	79.0	78.5	97.6	100.0				
NADL	75.3	75.9	75.5	77.9	78.5	79.1	78.5	80.4	80.3	79.8	93.9	92.6	100.0			
GIRAFFE_H1	76.5	77.2	76.7	77.9	80.4	80.4	76.1	73.6	73.5	73.6	77.3	77.9	78.5	100.0		
PRONGHORN	69.1	68.5	67.9	66.1	66.1	65.4	69.1	70.4	69.8	69.1	67.3	68.5	67.9	64.2	100.0	
BUNGOWANNAH	55.6	55.6	54.0	52.8	54.0	52.8	54.0	55.8	55.6	55.2	51.5	50.9	51.5	54.6	53.1	100.0

Table 4
Comparisons of the identity (%) of the novel virus with representative pestiviruses using the deduced amino acid sequence for the E2 coding region

	ALFORT	BRESCIA	PADERBORN	X818	T1802	V2536	REINDEER	NY_93_C	890	1373	CP7	NY_1	NADL	GIRAFFE_H1	PRONGHORN	BUNGOWANNAH
ALFORT	100.0															
BRESCIA	93.2	100.0														
PADERBORN	95.7	93.3	100.0													
X818	74.3	74.1	74.3	100.0												
T1802	74.3	73.8	74.1	90.9	100.0											
V2536	75.9	74.8	75.6	86.6	88.5	100.0										
REINDEER	74.9	75.1	75.1	83.4	85.8	84.5	100.0									
NY_93_C	62.3	61.2	61.5	64.2	63.9	63.3	65.5	100.0								
890	62.0	61.0	61.5	63.9	63.6	62.7	65.2	99.7	100.0							
1373	62.0	61.0	61.2	64.4	64.2	62.5	65.8	97.1	96.8	100.0						
CP7	59.1	55.4	59.1	57.5	57.5	58.6	58.0	65.3	64.3	65.3	100.0					
NY_1	67.8	66.8	66.8	67.6	67.6	67.3	66.0	72.4	71.9	72.7	88.6	100.0				
NADL	71.7	69.3	70.1	67.1	67.4	67.3	68.2	71.9	71.4	71.9	80.8	86.1	100.0			
GIRAFFE_H1	64.2	63.1	64.3	66.8	67.4	65.7	67.4	65.0	65.0	65.0	66.3	69.2	69.6	100.0		
PRONGHORN	54.3	52.9	55.1	55.6	56.4	55.2	54.3	54.3	54.6	55.1	53.9	54.2	55.9	55.9	100.0	
BUNGOWANNAH	50.8	48.7	49.9	50.3	51.3	49.9	49.2	50.8	50.3	51.3	47.7	50.7	48.3	48.7	47.6	100.0

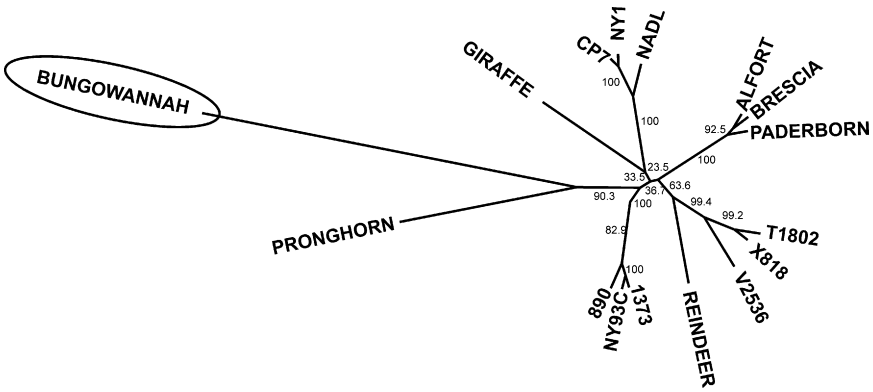

Fig. 2. Dendrogram comparing the genetic diversity of the novel virus with representative pestiviruses using the deduced amino acid sequence for the N^{pro} protein. Branch lengths are proportional to genetic distances. Numbers indicate the percentage of 1000 bootstrap replicates that support each labelled interior branch.

recognised with an additional species being considered (Thiel et al., 2005). Separation of pestiviruses into species has been controversial and considers several parameters. Examination of genome sequence relatedness, the presence of an N^{pro} coding region (which is unique to pestiviruses), antigenic relatedness by cross-neutralisation and other antigenic analyses and to a lesser extent host of origin and disease syndrome have each been considered when differentiating pestiviruses into species. Using these parameters, several additional genotypes have also been identified and some have been proposed as additional species within the genus. (Becher et al., 1997, 2003; Avalos Ramirez et al., 2001; Schirrmeier et al., 2004; Vilcek et al., 2005).

Currently, phylogenetic analysis of pestiviruses is based on sequences from the 5′UTR, N^{pro} and E2 regions (Becher et al., 1997, 2003; Vilcek et al., 2005) or, less frequently, the N^{pro}, E2 and NS3 regions (Schirrmeier et al., 2004).

While there is some sequence identity, the novel virus is only distantly related to viruses from any of the four currently defined pestivirus species. In addition, it does not cluster with the more divergent putative species, "pestivirus of giraffe". Although phylogenetic studies cluster it closer to the pronghorn antelope isolate than other viruses, sequence identity between these two viruses is only 62.8% based on the 5′UTR, 53.1% on the N^{pro} and 47.6% on the E2 protein. Thus, the sequence identity between the pronghorn virus and the novel virus is less than typically seen between the recognised pestivirus species. Overall, the novel virus is consistently more divergent than any previously recognised or proposed pestivirus species. Divergence by 37.2% to as much as 52.3% from any of the other pestiviruses warrants classification as a new species according to criteria proposed by Becher et al. (1999).

The results of the cell culture studies confirm that the viral sequence detected by SISPA represents part of the genome of a fully functional, competent pestivirus. The virus appears to have a predilection for porcine cells and has continued to replicate for many passages without a decline in titre. The results

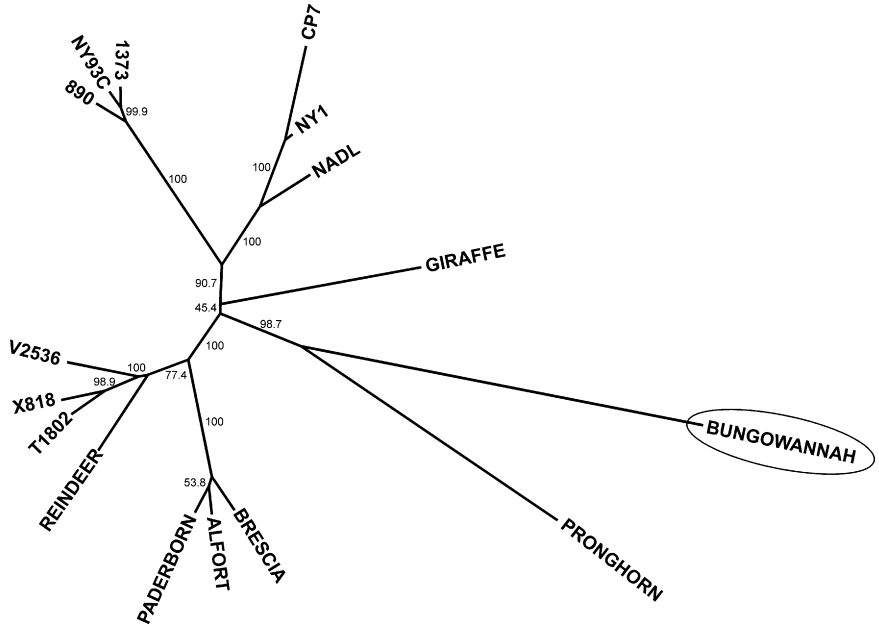

Fig. 3. Dendrogram comparing the genetic diversity of the novel virus with representative pestiviruses using the deduced amino acid sequence for the E2 protein. Branch lengths are proportional to genetic distances. Numbers indicate the percentage of 1000 bootstrap replicates that support each labelled interior branch.

of the IPX staining with the monoclonal antibodies and the virus neutralisation assays support the sequence data to show that this new virus is both genetically and antigenically highly divergent from the recognised members of the *Pestivirus* genus. The antigenic remoteness of this virus from other pestiviruses is demonstrated most forcefully by its failure to react with the pan-reactive monoclonal antibodies used in this study. In contrast, the same mAbs have been shown to react consistently with members of the recognised pestivirus species (*BVDV-1*, *BVDV-2*, *BDV*, *CSFV*) as well as the giraffe and 'HoBi' viruses (Dekker et al., 1995; Schirrmeier et al., 2004). While there is limited information on the antigenic characterisation of the pronghorn antelope virus, it did react with the only mAb used and neutralising activity was observed between this virus and strains of *BVDV-1*, *BVDV-2* and *BDV* (Vilcek et al., 2005). The novel virus that appears to be associated with the PMC syndrome has as yet not shown any evidence of cross neutralising activity with any of the other pestiviruses. However, in the broadly reactive AGID test that is believed to detect antibodies against the NS3 protein, there is clear evidence of cross reactivity with a *BVDV-1* virus, albeit weak reactivity. Antigens from other viruses have not been tested for cross-reactivity in this assay.

The antigens of this new virus also appear to be more readily affected by treatments used for cell fixation. Although the antigens in infected cell cultures are readily detected with the porcine convalescent serum after the fixation described, no staining could be detected if the monolayers were fixed by methods usually employed for other pestiviruses, including fixation with acetone or formalin/NP40 without prior freezing.

Collectively these data would suggest that this virus represents a previously undescribed species in the genus. Further studies are required to complete our knowledge of the entire genome of this agent and to support its classification as a probable new pestivirus species. Examination of field samples by PCR provided supporting evidence that this virus is present in the pig population affected by PMC. Studies to confirm that this virus is the causative agent for PMC are continuing.

Our success with the SISPA method in identifying another novel virus supports its role as a powerful technique to recognise and identify new agents. However, it would be beneficial if the method was further refined so that the large quantities of host DNA/RNA that are found in tissue samples do not compromise the identification of short viral sequences in affected organs.

In conclusion, this study has identified in pigs affected by the PMC syndrome a virus with a genome arrangement and nucleic acid sequences consistent with a pestivirus. We suggest that this is a novel pestivirus and that the data available would support its classification as a new pestivirus species. Finally, we propose the name "Bungowannah virus" for this novel pestivirus.

Acknowledgements

We are indebted to Dr. Royle Hawkes for his continuing encouragement and practical suggestions throughout this project and to other staff of the Virology Laboratory at EMAI for their technical assistance. Funding was generously provided by Australian Pork Limited and NSW Department of Primary Industries under Project No. 2001.

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